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Intraperiaqueductal Gray Glycine and D-Serine Exert Dual Effects on Rostral Ventromedial Medulla ON- and OFF-Cell Activity and Thermoceptive Threshold in the Rat

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Palazzo E, Guida F, Migliozzi A, Gatta L, Marabese I, Luongo L, Rossi C, de Novellis V, Fernández-Sánchez E, Soukupova M, Zafra F, Maione S. Intraperiaqueductal gray glycine and D-serine exert dual effects on rostral ventromedial medulla ON- and OFF-cell activity and thermoceptive threshold in the rat. *J Neurophysiol* 102: 3169–3179, 2009. First published September 23, 2009; doi:10.1152/jn.00124.2009. We have studied the involvement of the *N*-methyl-D-aspartate receptor (NMDAR) glycine site and the strychnine-sensitive glycine receptor (GlyR) in the ventrolateral periaqueductal gray (VL-PAG) on nociceptive behavior (tail flick) and pain-related changes on neuronal activity in the rostral ventromedial medulla (RVM). Glycine or D-serine increased the tail-flick latency, reduced OFF-cell pause, and delayed its onset and increased the time between the onset of the OFF-cell pause and the tail withdrawal. Conversely, they decreased the ongoing activity of the ON cell, the tail-flick–induced ON-cell firing, whereas they delayed the onset of increased tail-flick–induced ON-cell firing. Also, glycine or D-serine reduced the interval between the onset of the increased ON-cell firing and tail withdrawal. Whereas 7-Cl-kynurenic acid (7-Cl-KYN) prevented such effects, strychnine did not do so. A higher dose of 7-Cl-KYN or strychnine was per se able to reduce or increase tail-flick latency and increase or reduce ON-cell activities, respectively. A higher dose of glycine was hyperalgesic in the presence of 7-Cl-KYN, whereas such an effect was prevented by strychnine. These data suggest *1*) a dual role of glycine in producing hyperalgesia or analgesia by stimulating the GlyR or the NMDARs within the VL-PAG, respectively; *2*) consistently that RVM ON and OFF cells display opposite firing patterns to the stimulation of the VL-PAG NMDAR glycine site and GlyR activation; and *3*) a tonic role of these receptors within the VL-PAG–RVM antinociceptive descending pathway.

INTRODUCTION

It has been recently shown that glycine induces hyponociception when microinjected into the periaqueductal gray (PAG) (Martins et al. 2008), a well-known antinociceptive area whose stimulation evokes analgesia (Reynolds 1969). The analgesic effect of glycine within the PAG could appear controversial due to the fact that glycine is one of the most commonly used fast inhibitory neurotransmitters in the brain through the stimulation of specific strychnine-sensitive receptor chloride channels (GlyRs) (Lynch 2009).

However, together with D-serine, glycine also acts as an endogenous and obligatory coagonist of glutamate—the major excitatory neurotransmitter within the CNS at the *N*-methyl-D-aspartate receptor (NMDAR) glycine site (Hashimoto et al. 1993; Johnson and Ascher 1987; Parson et al. 1998; Schell et al. 1997). Thus glycine also acts as an excitatory neurotransmitter when it binds to the glycine site on the NMDAR. D-Serine, however, has a different pharmacological profile, since it binds only to the NMDAR glycine site. Having the potential to act either as an inhibitory or as an excitatory neurotransmitter, the site of action of glycine on NMDARs or GlyR at the PAG level is crucial in pain modulation. PAG is a key area of the antinociceptive descending pathway (Behbehani 1995; Fields 2000), which includes the rostral ventromedial medulla (RVM) as an intermediate station, projecting to the dorsal horn spinal cord (Basbaum and Fields 1984; Fields et al. 1995), thus regulating pain transmission.

The supraspinal levels of glycine and D-serine (De Miranda et al. 2002; Wolosker et al. 1999a,b) in the brain have been shown to be relatively higher (Hashimoto et al. 1995) than those that are necessary to stimulate the NMDAR and it is possible that, as shown for D-serine (Schell et al. 1995), much of the PAG glycine could act on the NMDARs. Although no conclusive evidence exists for supporting this hypothesis, or even to suggest any interaction of glycine on NMDARs, it is interesting that glycine transporter-1 (GLYT1) has been identified in the postsynaptic densities of asymmetric synapses (Cubelos et al. 2005), a sort of synaptic organization for concentrating glycine in the microenvironment around the NMDARs (Lim et al. 2004). However, most of the studies on glycine or D-serine regarding NMDAR modulation have been limited to in vitro systems; how and whether glycine and D-serine in the PAG may affect behavioral pain threshold and the activity of RVM neurons responding specifically to nociceptive stimuli are still poorly understood. In this study, we have aimed to further clarify the effect of intra-PAG microinjections of glycine or D-serine, either alone or in combination with strychnine or 7-Cl-kynurenic acid (7-Cl-KYN), in lightly anesthetized rats, on *1*) nociceptive reflexes to heat in the

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tail-flick test and *2*) spontaneous and tail-flick–related activities of Neutral, OFF, and ON cells of the RVM.

METHODS

Animals

In all, 280 Wistar male rats (250–300 g) were used in this study. Rats were housed 3 per cage under controlled illumination (12:12-h light:dark cycle; light on 6:00 am) and environmental conditions (ambient temperature: 20–22°C; humidity: 55–-60%) for ≥1 wk before the commencement of experiments. Rat chow and tap water were available without restriction. The experimental procedures were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with Italian Legislative Decree DL 116/92 and European Commission Directive L358/1, 18/12/86 regulations on the protection of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgical preparation for intra-PAG microinjections

To perform direct intra-ventrolateral (VL) PAG administrations of drugs or respective vehicle, artificial cerebrospinal fluid (ACSF, composition, in mM: KCl 2.5; NaCl 125; $MgCl_2$ 1.18; $CaCl_2$ 1.26), rats were anesthetized with pentobarbital (50 mg/kg, administered intraperitoneally) and a 26-gauge, 12-mm-long stainless steel guide cannula was stereotaxically lowered until its tip was 1.5 mm above the ventrolateral PAG by applying coordinates from the atlas of Paxinos and Watson (1986) (anterior [A]: −7.8 mm; lateral [L]: 0.5 mm from bregma; ventral [V]: 4.3 mm below the dura). VL-PAG was considered in this study, since previous studies have shown the presence of excitatory output neurons projecting to OFF neurons in the RVM in that area (Moreau and Fields 1986; Sandkulher and Gebhart 1984). The cannula was anchored with dental cement to a stainless steel screw in the skull. We used a David Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), with the animal positioned on a homeothermic temperature-controlled blanket (Harvard Apparatus, Edenbridge, Kent, UK). Direct intra-VL-PAG administration of drugs, or respective vehicle, was conducted with a stainless steel cannula connected by a polyethylene tube to a 1-μl 31-gauge syringe (SGE Analytical Science, Austin, TX), inserted through the guide cannula and extended 1.5 mm beyond the tip of the guide cannula to reach the VL-PAG. Volumes of 200-nl drug solutions, or vehicle, were injected into the VL-PAG over a period of 60 s and the injection cannula was gently removed 2 min later.

RVM extracellular recordings and tail-flick test

After implantation of the guide cannula into the VL-PAG (see preceding section), a tungsten microelectrode was stereotaxically lowered through a small craniotomy into the RVM (anterior–posterior [AP]: −11.5; L: +0.3; V: 9.9–10.9) (Paxinos and Watson 1986) to record the activity of Neutral, ON, and OFF cells. The OFF cells were identified by the characteristic pause when noxious stimulation was applied (Fields et al. 1983; Heinricher et al. 1989) and the criterion used for the onset of the OFF-cell pause was the interval between the onset of heat application and the last action potential prior to the tail flick. With respect to ON cells, they have been identified by a sudden increased firing (spikes/s) beginning just prior to the occurrence of the reflex. Neutral cells did not show any significant change in activity associated with the tail withdrawal (Morgan et al. 2008). Anesthesia was maintained with a constant, continuous infusion of propofol (5–10 $mg \cdot kg^{-1} \cdot h^{-1}$, intravenous). Anesthesia was adjusted so that tail flicks were elicited with a constant latency of 4–5 s. A thermal stimulus was elicited by a radiant heat source of a tail-flick unit (model 7360; Ugo Basile, Varese, Italy) focused on the rat tail about 3–5 cm from the tip. The rat tail was placed over the surface of a slightly projecting window receiving the infrared (IR) energy. Tail-flick latency in seconds was determined by a timer connected to a photoelectric cell that stopped the timer (and switched off the lamp) upon movement of the tail, which was withdrawal. By using a rheostat, the intensity of current through the filament and therefore of the radiant heat emission could be controlled (Le Bars et al. 2001) and adjusted at the beginning of each experiment to elicit a constant tail-flick latency. The IR intensity in our experiments was set to 50 mW, corresponding to 50 mJ/s (1 mW in 1 s produces an energy of 1 mJ^{-1}; a joule can be transformed into calories by using the conversion factor 0.239). Tail flicks were elicited every 5 min for ≥15 min (or 20 min only in the experiments with 10-min strychnine pretreatment) prior to microinjecting drugs, or respective vehicle, into the VL-PAG. Tail-flick latencies were monitored in the same rats subjected to RVM ON- and OFF-cell recordings.

Extracellular single-unit recordings were made in the RVM with glass-insulated tungsten filament electrodes (3–5 MΩ; FHC, Bowdoin, ME). The recorded signals were amplified and displayed on a digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were also fed into a window discriminator whose output was processed by an interface CED 1401 (Cambridge Electronic Design [CED], Cambridge, UK) connected to a Pentium III PC. Spike2 software (CED, version 4) was used to create peristimulus rate histograms on-line and to store and analyze digital records of single-unit activity off-line. Configuration, shape, and height of the recorded action potentials were monitored and recorded continuously using a window discriminator and Spike2 software for on-line and off-line analysis. Once a Neutral, ON, or OFF cell was identified, we optimized spike size before all treatments. This study included only neurons whose spike configuration remained constant and could clearly be discriminated from activity in the background throughout the experiment, indicating that the activity from only one neuron was measured. Only one neuron was recorded from each rat.

Histology

At the end of the experiment, a volume of 200 nl of neutral red (0.1%) was also injected into the VL-PAG 30–40 min before killing the rat. Rats were then intracardially perfused with 200 ml phosphate buffer solution (PBS) followed by 200 ml 10% formalin solution in PBS. The brains were removed and immersed in a saturated formalin solution for 2 days. The injection site was ascertained by using two consecutive sections (40 μm), one stained with cresyl violet to identify nuclei and the other unstained to determine dye spreading. Only those rats whose microinjected site was located within the VL-PAG were used for data computation.

Recording sites were recognized with an electrolytic lesion at the conclusion of the experiment. The rats were killed with an overdose of pentobarbital and perfused with 10% formalin. Locations of all the studied neurons were reconstructed and plotted on standardized sections. Cells located outside the RVM were excluded from the study.

Treatments

Groups of six to eight animals for the ON- and six to eight for the OFF-cell recordings were used for each treatment, with each animal used for a single-cell recording.

Rats receiving intra-VL-PAG administration of vehicle or different doses of glycine or D-serine, alone or in combination with 7-Cl-KYN or stychnine, were grouped as follows.

1. A group of rats was implanted with guide cannulae and received an intra-VL-PAG microinjection of 200 nl of ACSF and served as a control for the intra-PAG drug microinjections.

2. Groups of rats received intra-VL-PAG microinjection of glycine (5 and 10 nmol), 7-Cl-KYN (1 and 2 nmol), or strychnine (1 and 2

nmol) alone. Glycine (10 nmol) was also administered in combination with 7-Cl-KYN (1 nmol) or strychnine (1 nmol). Additional groups of rats received intra-VL-PAG microinjection of glycine (20 nmol) either alone or coinjected with 7-Cl-KYN (1 nmol). Moreover, another group of rats received glycine (20 nmol) in combination with 7-Cl-KYN (1 nmol) 10 min after strychnine (1 nmol).

3. Groups of rats received an intra-VL-PAG microinjection of D-serine (2 and 4 nmol) alone or D-serine (4 nmol) in combination with 7-Cl-KYN (1 nmol) or strychnine (1 nmol). Additional groups of rats received an intra-VL-PAG microinjection of a higher dose of D-serine (8 nmol), either alone or in combination with 7-Cl-KYN (1 nmol).

The doses of glycine, D-serine, or antagonists for intra-VL-PAG were chosen on the basis of other previous in vivo studies (Berrino et al. 1993; Martins et al. 2008; Matheus et al. 1994; Santos et al. 2006) as well as our own. In particular, since to our knowledge no other study has been published describing the effects of strychnine by using a similar administration route in the PAG, we performed preliminary experiments with several doses of strychnine to find minimal doses able to change RVM cell activities or tail-flick latencies.

Drugs

7-Cl-kynurenic acid (7-Cl-KYN) and strychnine were purchased from Tocris Cookson (Bristol, UK). Glycine and D-serine were purchased from Sigma. All drugs were dissolved in ACSF, with final pH 7.2 for intra-PAG microinjection.

Statistics

The neuron responses, before and after intra-VL-PAG vehicle or drug microinjections, were measured and expressed as spikes/s (Hz). Background activity of neurons was measured between tail flicks. In particular, basal values were obtained by averaging the activities recorded in 50 s before the application of three consecutive thermal stimulations (each stimulation trial was performed every 5 min). Four thermal stimulations were considered only in the set of experiments with 10-min strychnine pretreatment. Data are presented as means ± SE in neuron responses either of changes in withdrawal latencies (s, tail-flick test) or changes in neuron response (extracellular recordings). Tail-flick–related ON-cell firing was calculated as the number of spikes in the 2-s interval beginning 0.5 s before the tail flick, before and after drug treatments. Furthermore, the time between the beginning of ON-cell increase in frequency and the tail withdrawal was calculated (s). The onset of the ON-cell burst was also calculated as the time elapsed between the onset of the applied noxious radiant heat and the beginning of the tail-flick–related increase in the cell frequency, which was at least double that of baseline spontaneous activity.

We also performed analysis of tail-flick–related OFF-cell activities before and after drug treatments. The latency to onset of the OFF-cell pause (the interval between the onset of thermal stimulus and the last spike) and the duration of the cell pause (the interval between the pause onset and the first spike after the tail flick) were determined. The interval between the onset of the ON-cell pause and the tail withdrawal was also calculated (s).

Comparisons between pre- and post-treatment ongoing and tail-flick–related cell activity changes were performed by repeated-measures ANOVA. Comparisons between groups of rats with different treatments were performed by using Wilcoxon signed-ranks test. $P < 0.05$ was considered statistically significant.

RESULTS

Glycine and D-serine increased tail-flick latencies

Tail flicks were elicited every 5 min for ≥15 min prior to microinjecting drugs or respective vehicle into the VL-PAG. Data related to pretreatment intervals were considered as basal tail-flick latencies (5.2 ± 0.4 s). Intra-VL-PAG microinjection of vehicle did not change the tail-flick latency compared with basal values (4.9 ± 0.7 s, $n = 20$) (Fig. 1). Tail-flick latency was significantly increased to 7.3 ± 0.5 ($P < 0.05$, $n = 15$) and 9.4 ± 0.7 s ($P < 0.05$, $n = 14$) or to 6.7 ± 0.3 ($P < 0.05$, $n = 15$) and 8.6 ± 0.6 s ($P < 0.05$, $n = 12$) by intra-VL-PAG microinjections of glycine (5 and 10 nmol) (Fig. 1*A*) or D-serine (2 and 4 nmol) (Fig. 1*B*), respectively.

Coinjection with 7-Cl-KYN (1 nmol) prevented the effects of glycine (10 nmol) ($P < 0.05$, $n = 16$) (Fig. 1*A*) and D-serine (4 nmol) ($P < 0.05$, $n = 14$) (Fig. 1*B*). Strychnine (1 nmol)

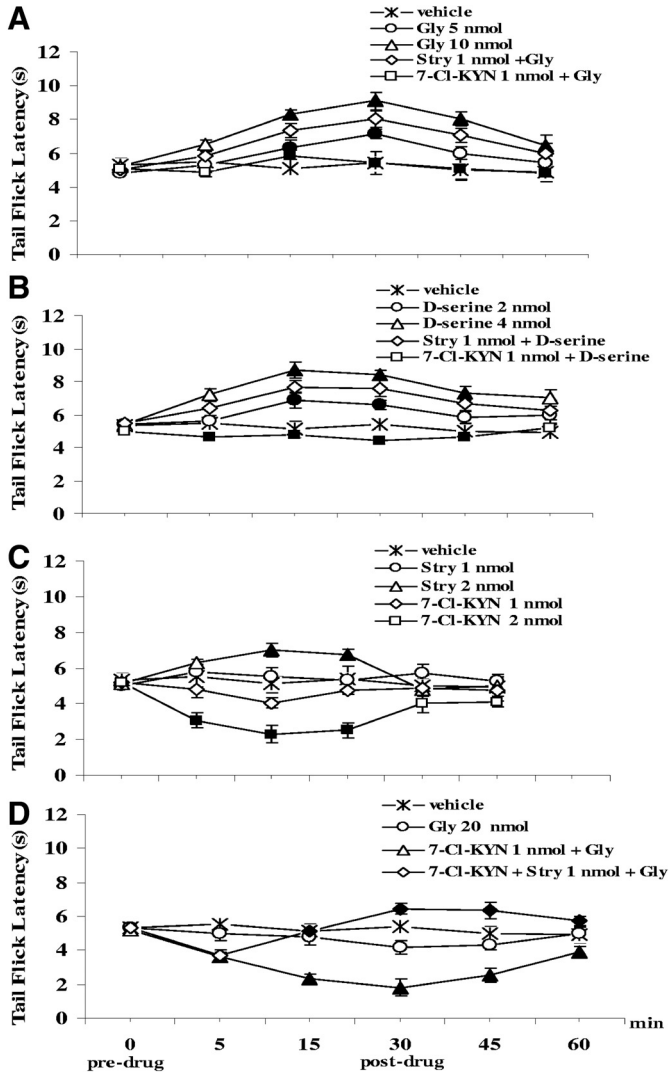

FIG. 1. Tail-flick latencies before (predrug) and after (postdrugs) microinjections into the ventrolateral-periaqueductal gray (VL-PAG) of vehicle, glycine (Gly), D-serine, strychnine (Stry), and 7-Cl-kynurenic acid (7-Cl-KYN). *A*: the effects of glycine (5 and 10 nmol), or glycine (10 nmol) in combination with strychnine (1 nmol) or 7-Cl-KYN (1 nmol). *B*: the effects of vehicle, D-serine (2 and 4 nmol), or D-serine (4 nmol) in combination with strychnine (1 nmol) or 7-Cl-KYN (1 nmol). *C*: the effects of vehicle, strychnine (1 and 2 nmol), or 7-Cl-KYN (1 and 2 nmol). *D*: the effects of glycine (20 nmol) alone or in combination with 7-Cl-KYN (1 nmol) or glycine in combination with 7-Cl-KYN after pretreatment with strychnine (1 nmol). Each point represents the mean ± SE of 12–20 observations. Filled symbols indicate significant differences vs. vehicle, glycine (10 nmol) (*A*), D-serine (4 nmol) (*B*), or glycine in combination with 7-Cl-KYN (*D*). Values of $P < 0.05$ were considered statistically significant.

failed to antagonize the effects of glycine (10 nmol, $n = 16$) (Fig. 1*A*) and D-serine (4 nmol, $n = 14$) (Fig. 1*B*).

High doses of 7-Cl-KYN and strychnine decreased or increased tail-flick latency, respectively

With respect to the effects of the highest doses of the antagonists, we found that tail-flick latency significantly increased to 7.2 ± 0.5 s ($P < 0.05$, $n = 14$) with intra-VL-PAG microinjection of strychnine (2 nmol), whereas it decreased to 2.8 ± 0.5 s with 7-Cl-KYN (2 nmol) ($P < 0.05$, $n = 14$) (Fig. 1*C*).

A high dose of glycine (20 nmol) did not significantly change tail-flick latency (4.2 ± 0.3 s, $n = 15$). Moreover, tail-flick latency significantly decreased to 2.4 ± 0.8 s with an intra-VL-PAG microinjection of glycine (20 nmol) in combination with 7-Cl-KYN (1 nmol) ($P < 0.05$, $n = 15$) (Fig. 1*D*). These latter effects were prevented by pretreatment with strychnine (1 nmol) ($P < 0.05$, $n = 16$).

Glycine or D-serine decreased or increased the ongoing activities of RVM ON and OFF cells, respectively

The results are based on RVM neurons at a depth of 8,870–10,540 μm from the surface of the brain, the location of the neurons being in the nucleus raphe magnus, nucleus reticularis gigantocellularis pars α, and nucleus reticularis paragigantocellularis (Fig. 2*B*). All recorded neurons were spontaneously active and discharged with a mean frequency (in spikes/s) of 6.3 ± 0.8 (Neutral cells), 7.08 ± 0.4 (ON cells), and 8.1 ± 0.5 (OFF cells). These neurons were identified by the characteristic OFF-cell pause and ON-cell burst of activity just before tail-flick responses or by their nonresponsiveness to noxious stimuli for the Neutral cells. Microinjections of glycine (5–10 nmol) into the VL-PAG caused a dose-dependent decrease in the firing activity of the ON cells, which was significant between 10 and 40 min and maximal 25 min after administration of the 10 nmol ($P < 0.05$, $n = 7$) (Fig. 3*A*). The same treatment produced a very rapid significant increase in the firing activity of the OFF cells, which was already significant after 5 min and maximal after 15 min from administration of the 10 nmol ($P < 0.05$, $n = 7$) (Fig. 3*B*). Similarly to glycine, microinjections of D-serine (2 and 4 nmol) into the VL-PAG caused a dose-dependent decrease in the firing activity of the ON cells that was significant between 15 and 40 min and maximal 25 min after administration of the 4 nmol ($P < 0.01$, $n = 6$) (Fig. 3*C*) as well as a very rapid increase in the firing activity of the OFF cells (Fig. 3*D*), which was significant between 10 and 35 min and maximal 25 min after administration of the 4 nmol dose ($P < 0.01$, $n = 6$) (Fig. 3*D*). Spontaneous activities of RVM Neutral neurons ($n = 6$), as identified by their nonresponsiveness to tail flick, were also analyzed before and after intra-VL-PAG microinjection of glycine (10 nmol) or D-serine (4 nmol), which failed to cause any change in their spontaneous activity (6.5 ± 0.7, $n = 6$ and 6.1 ± 0.5 spike/s, $n = 6$, respectively) (Fig. 4*A*). Finally, microinjection of glycine (10 nmol) outside the VL-PAG (Fig. 2*A*) did not change the ON-cell (7.1 ± 0.4 spikes/s) and OFF-cell (7.9 ± 0.6 spike/s) spontaneous activity.

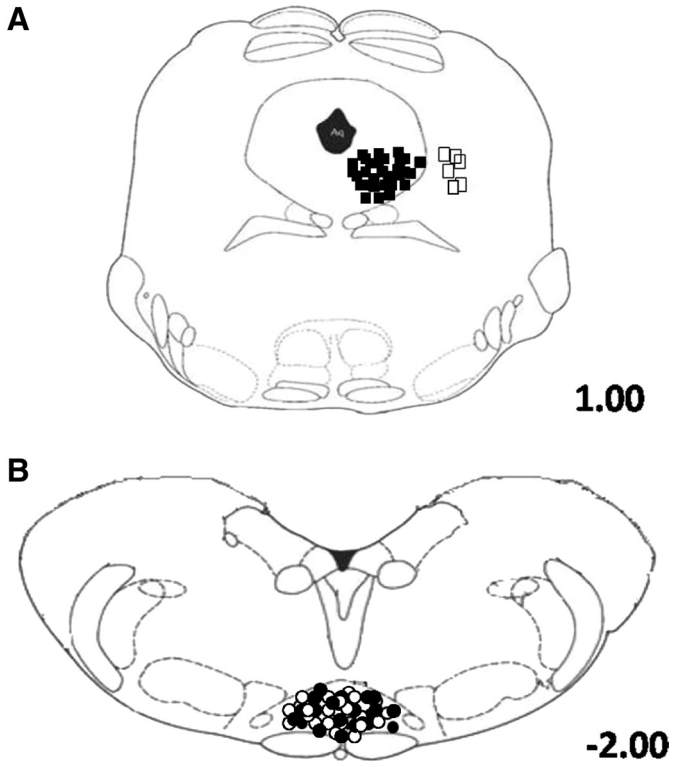

FIG. 2. Schematic illustration of the location of microinjection sites (*A*) and rostral ventromedial medulla (RVM) ON- or OFF-cell recording sites (*B*). Vehicle or drug microinjections were performed in the VL-PAG (filled squares) (*A*). Open squares indicate the microinjection sites performed outside the VL-PAG, which were associated neither with change in RVM cell activity nor with tail-flick latency. Moreover, cell recordings were performed by lowering a tungsten electrode into the RVM and ON-cell (filled circles) or OFF-cell (open circles) sites (*B*) are shown. Many sites are not shown to avoid overlapping of symbols. Distances (in mm) from the interaural line are indicated.

7-Cl-KYN prevented the effect of glycine or D-serine on RVM ON- and OFF-cell ongoing activity, whereas strychnine did not

The electrophysiological effects induced by glycine or D-serine were significantly prevented when 7-Cl-KYN (1 nmol), the antagonist of the NMDAR glycine site, was coinjected into the VL-PAG (Fig. 3). In particular the coinjection with 7-Cl-KYN (1 nmol) significantly prevented the effect of glycine (10 nmol) on the ON cell, an effect that started after 10 min and lasted for the entire recording period ($P < 0.05$, $n = 8$) (Fig. 3*A*). The coinjection with 7-Cl-KYN (1 nmol) significantly prevented the effect of glycine (10 nmol) on the OFF-cell ongoing activity, an effect that was significant between 5 and 35 min ($P = 0.01$ at 30 min postinjection, $n = 8$) (Fig. 3*B*). Moreover, the coinjection with 7-Cl-KYN (1 nmol) significantly prevented the effect of D-serine (4 nmol) on the ON cells. This effect was significant between 15 and 40 min ($P < 0.01$ at 15 min postdrug coinjection, $n = 7$) (Fig. 3*C*). The coinjection with 7-Cl-KYN (1 nmol) significantly prevented the effect of D-serine on the ongoing activity of OFF cells, an effect that was significant between 10 and 35 min ($P < 0.01$ at 15 min, $n = 7$) (Fig. 3*D*). The effects of glycine or D-serine were not prevented by strychnine (1 nmol), the selective GlyR antagonist (Fig. 3).

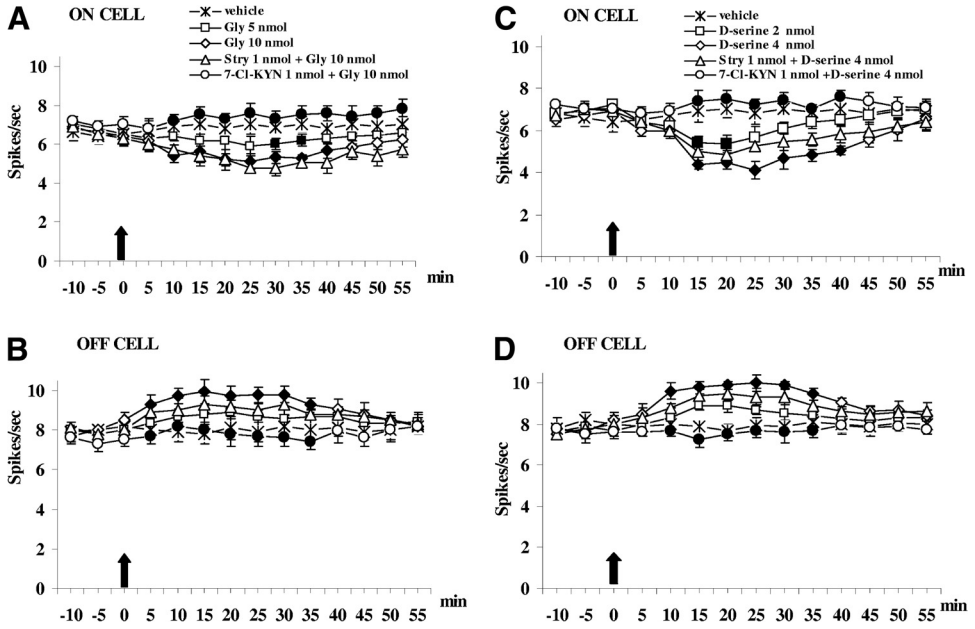

FIG. 3. Effect of vehicle, glycine, and D-serine on the spontaneous firing of RVM ON (*A*, *C*) or OFF (*B*, *D*) cells. *A* and *B*: the effect of intra-VL-PAG administration of vehicle, glycine (5 and 10 nmol), or glycine (10 nmol) in combination with strychnine (1 nmol) or 7-Cl-KYN (1 nmol). *C* and *D*: the effect of intra-VL-PAG administration of D-serine (2 and 4 nmol) alone or D-serine (4 nmol) in combination with strychnine (1 nmol) or 7-Cl-KYN (1 nmol). The arrows indicate drug microinjections. Each point represents the mean ± SE of 6–8 neurons. Filled symbols indicate significant differences vs. vehicle and vs. Gly (10 nmol) (*A*, *B*) and D-serine (4 nmol) (*C*, *D*). *P* values <0.05 were considered statistically significant.

High doses of 7-Cl-KYN and strychnine conversely modified the ongoing ON- and OFF-cell activity

Microinjection of a higher dose of 7-Cl-KYN (2 nmol) into the VL-PAG caused an increase in the firing activity of the ON cells, which was significant between 5 and 35 min and maximal 20 min after administration ($P < 0.01$, $n = 7$) (Fig. 4*B*). It also caused a very rapid decrease in the firing activity of the OFF cells, significant after 5 min and maximal 20 min after drug administration ($P < 0.01$, $n = 7$) (Fig. 4*C*). Microinjections of a higher dose of strychnine (2 nmol) into the VL-PAG caused a decrease in the ongoing activity of the ON cells, which was significant between 10 and 30 min and maximal 15 min after administration ($P < 0.01$, $n = 7$) (Fig. 4*B*). Strychnine also caused a very rapid, significant increase in the firing activity of the OFF cells, which was maximal 15 min after the administration of strychnine ($P < 0.05$, $n = 7$) (Fig. 4*C*).

Effect of a high dose of glycine (but not of D-serine) on the RVM cell firing was enhanced by 7-Cl-KYN and prevented by strychnine

Microinjections into the VL-PAG of the highest dose of glycine (20 nmol) caused a slight increase in the firing activity of the ON cells, which was significant between 25 and 30 min and maximal 25 min after administration ($P < 0.05$, $n = 7$) (Fig. 5*A*). Glycine (20 nmol) also induced a decrease in the firing activity of the OFF cells that was significant between 15 and 35 min and maximal at 20 min ($P < 0.05$, $n = 7$) (Fig. 5*B*). These electrophysiological effects were accompanied by transient hyperalgesia, as measured with the tail-flick test (see the preceding text). The effect of glycine (20 nmol) was significantly enhanced when administered in combination with 7-Cl-KYN (1 nmol). The effect on the ON cells was significant between 5 and 35 min and maximal 15 min after administration ($P < 0.01$, $n = 8$) (Fig. 5*A*). The effect on the OFF cells was significant between 10 and 40 min and maximal 20 min after administration ($P = 0.01$, $n = 7$) (Fig. 5*B*). Pretreatment with strychnine (1 nmol) significantly prevented these effects on the ON ($P < 0.01$, $n = 8$) and OFF cells ($P < 0.01$, $n = 8$) (Fig. 5, *A* and *B*).

Conversely, microinjection into the VL-PAG of the highest dose of D-serine (8 nmol) decreased the firing activity of the ON cells, which was significant between 5 and 30 min and maximal 10 min after administration ($P < 0.05$, $n = 7$). It increased the OFF-cell firing activity, which was significant between 10 and 20 min and maximal at 15 min ($P < 0.05$, $n = 6$) (not shown). These electrophysiological effects were accompanied by analgesia measured by the tail-flick test (not shown).

Glycine or D-serine modified tail-flick–related RVM cell activities and tail withdrawal

Glycine and D-serine both modified tail-flick–related ON- and OFF-cell activity. Glycine (5 nmol) significantly ($P < 0.05$, $n = 7$) changed the onset of the ON-cell burst (3.3 ± 0.4 vs. 5.7 ± 0.5 s), whereas glycine (10 nmol) strongly reduced the ON-cell burst activity (from 14.5 ± 3.7 to 5.2 ± 1.4 spikes/s; $P < 0.05$, $n = 7$) (Fig. 6*E*) and significantly decreased the duration of the OFF-cell pause (from 6.5 ± 1.7 to 2.1 ± 0.3 s) ($P < 0.05$, $n = 7$) (Fig. 6*E*). Glycine (10 nmol) also delayed the onset of OFF-cell pause (from 2.8 ± 0.4 to 6.5 ± 0.7 s; $P < 0.05$, $n = 7$). D-Serine (4 nmol) significantly delayed the onset of the ON-cell burst (2.6 ± 0.4 vs. 5.3 ± 0.6 s; $P < 0.05$, $n = 6$) and shortened the time between the onset burst of the ON cell and tail withdrawal latency (1.2 ± 0.6 vs. 0.6 ± 0.4 s; $P < 0.05$, $n = 6$), whereas it significantly decreased the duration of the OFF-cell pause (from 6.2 ± 1.1 to 2.4 ± 0.4 s; $P < 0.05$, $n = 6$) (Fig. 6*F*). D-Serine (4 nmol) significantly inhibited tail-flick–induced ON-cell peak firing (from 16.5 ± 2.4 to 4.2 ± 0.6 spikes/s; $P < 0.05$, $n = 6$) (Fig. 6*F*), delayed the onset of OFF-cell pause (from 2.9 ± 0.6 to 6.2 ± 0.8 s; $P < 0.05$, $n = 6$), and delayed the time between the onset of the OFF-cell pause and the tail withdrawal (from 1.1 ± 0.5 to 1.9 ± 0.4 s; $P < 0.05$, $n = 6$) (Fig. 6).

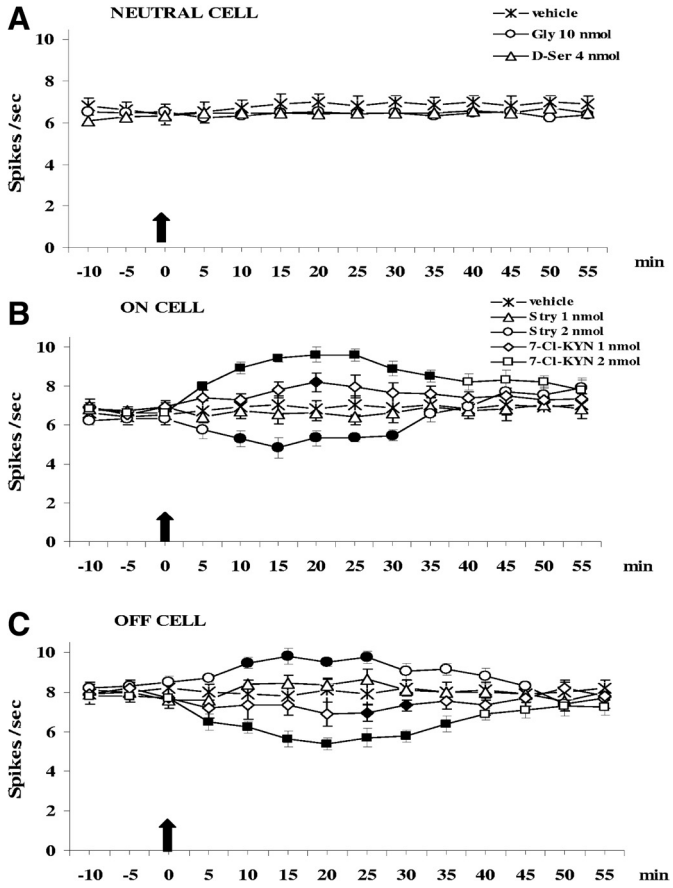

FIG. 4. Effect of vehicle, glycine (10 nmol), or D-serine (4 nmol) on the spontaneous firing of RVM neutral cells (*A*) or the effect of vehicle, strychnine (1 and 2 nmol), or 7-Cl-KYN (1 and 2 nmol) on the ON (*B*) or OFF (*C*) cell ongoing activity. The arrows indicate drug microinjections. Each point represents the mean ± SE of 6–8 neurons. Filled symbols indicate significant differences vs. vehicle. *P* values <0.05 were considered statistically significant.

Coadministration of a high dose of glycine with 7-Cl-KYN modified tail-flick–related ON- and OFF-cell activity in a way prevented by strychnine

The highest dose of glycine (20 nmol) did not modify the tail-flick–related ON- and OFF-cell activity, whereas, in combination with 7-Cl-KYN, it shortened the onset of the ON-cell burst (3.1 ± 0.4 vs. 1.5 ± 0.4 s; $P < 0.05$, $n = 8$) and increased the duration of the OFF-cell pause (from 3.2 ± 1.2 to 5.9 ± 0.6 s; $P < 0.05$, $n = 7$). Microinjection of glycine (20 nmol) in combination with 7-Cl-KYN also increased tail-flick–induced ON-cell peak firing (from 10.5 ± 6.2 to 17.5 ± 2.8 spikes/s; $P < 0.05$, $n = 8$) and shortened the onset of OFF-cell pause (from 3.3 ± 0.4 to 2.1 ± 0.3 s; $P < 0.05$, $n = 7$). This glycine-induced effect in combination with 7-Cl-KYN was prevented by pretreatment with strychnine (1 nmol) (data not shown). Figure 6 shows examples of ratemeter records that illustrate the effect of intra-VL-PAG microinjection of glycine (10 nmol) and D-serine (4 nmol) on either the ongoing activity or tail-flick–related burst of activity of an identified RVM ON cell (Fig. 6, *A* and *C*) and the ongoing activity or tail-flick–related pause of an identified RVM OFF cell (Fig. 6, *B* and *D*).

Highest doses of 7-Cl-KYN or strychnine modified tail-flick–related changes on RVM cell activities

The highest doses of 7-Cl-KYN (2 nmol) or strychnine (2 nmol) used here modified tail-flick–related ON- and OFF-cell activity (Fig. 7). 7-Cl-KYN (2 nmol) significantly shortened the onset of the ON-cell burst (2.6 ± 0.5 vs. 1.5 ± 0.4 s, $P < 0.05$, $n = 7$) and delayed the time between the onset of the ON-cell burst and the tail withdrawal (0.9 ± 0.4 vs. 1.8 ± 0.5 s; $P < 0.05$, $n = 7$), whereas it increased the duration of the OFF-cell pause (from 5.1 ± 1.7 to 8.7 ± 0.9 s; $P < 0.05$, $n = 7$) (Fig. 7*E*). 7-Cl-KYN also significantly increased tail-flick–induced ON-cell peak firing (from 12.9 ± 3.2 to 19.5 ± 2.3 spikes/s; $P < 0.05$, $n = 7$) (Fig. 7*E*), although it also shortened the onset of OFF-cell pause (from 2.7 ± 0.4 to 1.5 ± 0.4 s; $P < 0.05$, $n = 7$) and shortened the interval between the onset of the OFF-cell pause and the tail withdrawal (from 1.8 ± 0.3 to 0.7 ± 0.4 s; $P < 0.05$, $n = 7$).

Strychnine (2 nmol) significantly increased the ON-cell onset of burst (3.5 ± 0.5 vs. 6.7 ± 0.8 s, $P < 0.05$, $n = 7$) and shortened the time between the onset of the ON-cell burst and the tail withdrawal (1.5 ± 0.6 vs. 0.8 ± 0.3 s; $P < 0.05$, $n = 7$), while decreasing the duration of the OFF-cell pause (from 5.8 ± 2.2 to 2.5 ± 0.6 s; $P < 0.05$, $n = 7$) (Fig. 7*F*). Moreover, strychnine decreased tail-flick–induced ON-cell peak firing (from 13.2 ± 4 to 7.4 ± 2.5 spike/s, $P < 0.05$, $n = 7$) (Fig. 7*F*), delayed the onset OFF-cell pause, and delayed the time

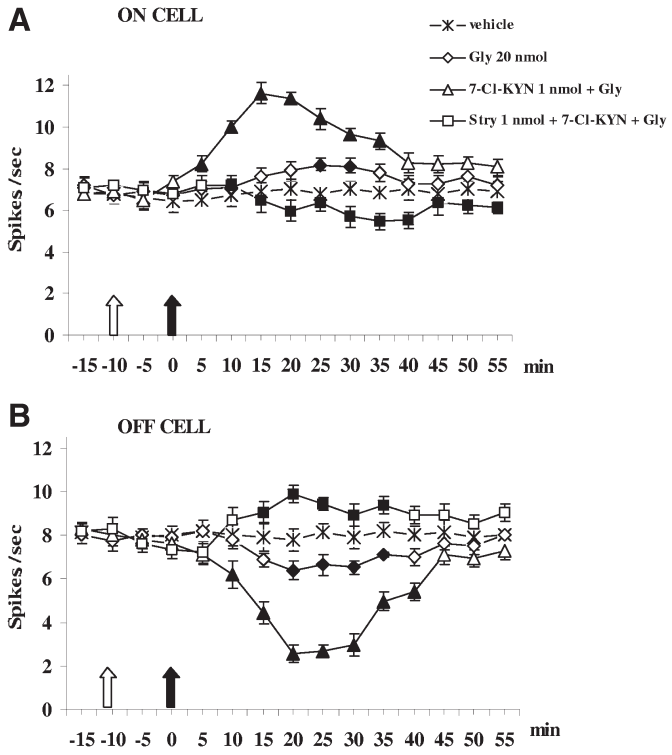

FIG. 5. Effect of vehicle, glycine (20 nmol) alone or in combination with 7-Cl-KYN (1 nmol), or Gly and 7-Cl-KYN in combination with strychnine (1 nmol) on RVM ON (*A*) and OFF (*B*) spontaneous cell firing. The black arrow indicates the administrations of vehicle, glycine, or glycine in combination with 7-Cl-KYN; the white arrow indicates strychnine 10 min pretreatment. Each point represents the mean ± SE of 6–8 neurons. Filled symbols indicate significant differences vs. vehicle vs. Gly (20 nmol) alone or in combination with 7-Cl-KYN (1 nmol). *P* values <0.05 were considered statistically significant.

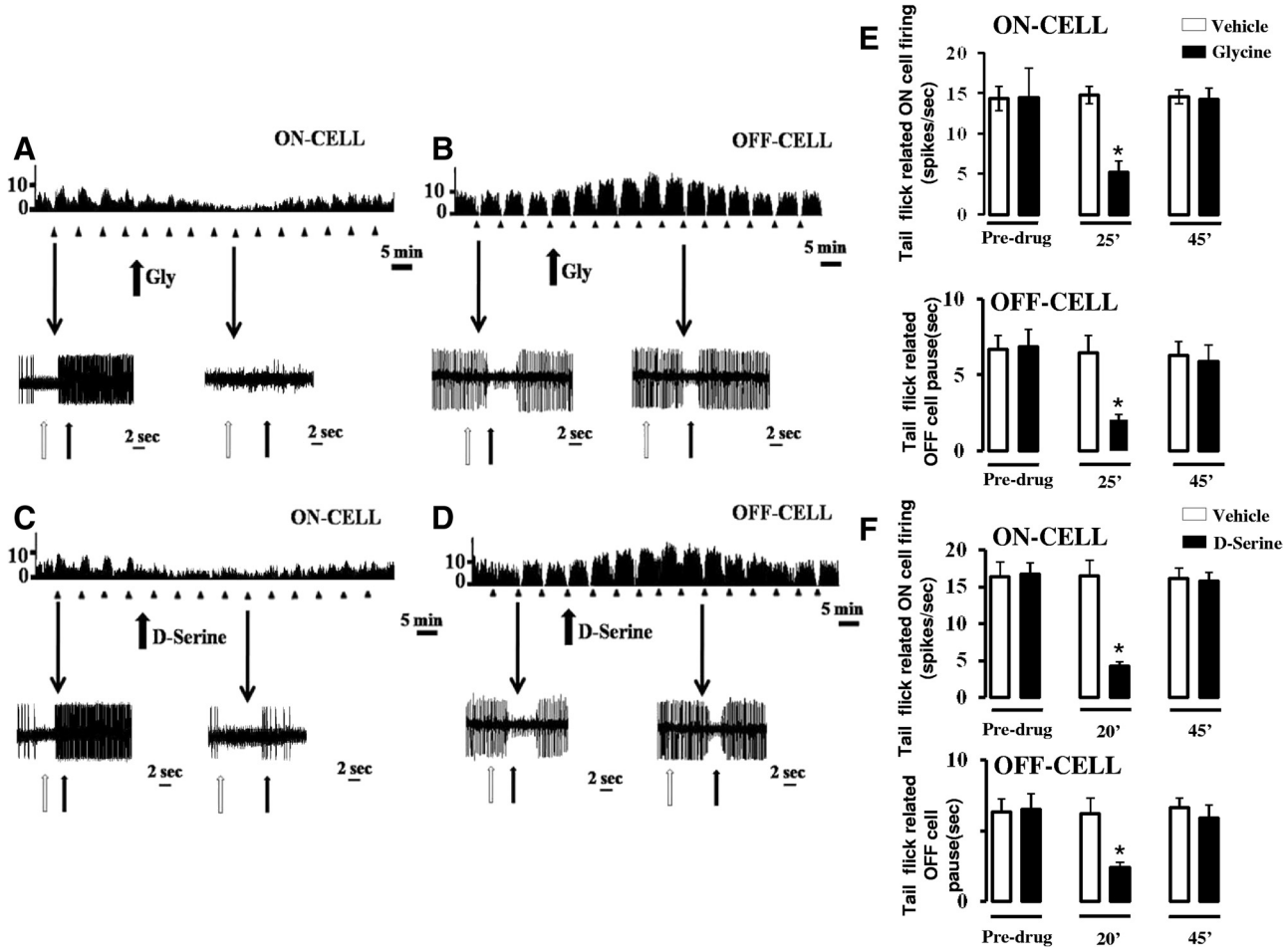

FIG. 6. Examples of ratemeter records that illustrate the effect of intra-PAG microinjection of glycine (10 nmol) (*A*, *B*) and D-serine (4 nmol) (*C*, *D*) on either the ongoing or tail flick-related burst of activity of identified RVM ON cell (*A*, *C*) and ongoing or tail-flick–related pause of identified RVM OFF cell (*B*, *D*). Intra-PAG microinjection of glycine (10 nmol) and D-serine (4 nmol) reduced the ongoing activity and the tail-flick–related burst of the ON cell (*A*, *C*). The same treatments increased the ongoing activity and reduced the tail-flick–related pause of the OFF cell (*B*, *D*). Single oscilloscope traces, indicated by the long black arrows, show the effect of tail-flick stimulation (small gray arrows) and tail reflex (small black arrows) on the ON cell increased frequency or OFF cell pause before and 20 min after intra-PAG glycine (10 nmol) and D-serine (4 nmol) microinjections. Filled triangles indicate tail flick trials, 1-s bins. Scale bars indicate 5-min or 2-s intervals for ratemeter records or single oscilloscope traces, respectively. *E* and *F*: population data (means ± SE) related to the ON-cell firing (Hz) and OFF-cell pause (s) after microinjection of glycine (*E*) or D-serine (*F*).

between the onset of the OFF-cell pause and the tail withdrawal (0.9 ± 0.3 to 1.8 ± 0.7 s, $P < 0.05$, $n = 7$). Figure 7 shows examples of ratemeter records that illustrate the effect of intra-VL-PAG microinjection of 7-Cl-KYN (2 nmol) and strychnine (2 nmol) on either the ongoing activity or tail-flick–related burst of activity of an identified RVM ON cell (Fig. 7, *A* and *C*) and ongoing activity or tail-flick–related pause of an identified RVM OFF cell (Fig. 7, *B* and *D*).

DISCUSSION

Thermal nociceptive responses and in vivo electrophysiological findings in this study provide evidence that glycine and D-serine—endogenous coagonists at the strychnine-insensitive glycine-recognition site on the NMDAR (Furukawa and Gouaux 2003; Johnson and Ascher 1987)—can modulate pain at the level of the VL-PAG in the rat. VL-PAG–RVM circuitry constitutes a main part of the endogenous antinociceptive pathway that inhibits pain. Different pain-responding neurons are found in the RVM: pain stimuli increase ON-, inhibit OFF-, and do not affect Neutral-cell activity (Fields and Basbaum 1994; Fields et al. 1991). The ongoing and tail-flick–related activities of these cells, with particular regard to ON and OFF cells, represent a suitable target to investigate the effect of pain-modulating agents at the PAG level.

Interestingly, we observed that glycine exerted a dual dose-dependent effect on RVM ON- and OFF-cell activity and on thermoceptive threshold in the same rat, whereas D-serine did not have such a dual effect. More specifically, we found that, on the one hand, if D-serine always induced dose-dependent and strychnine-insensitive analgesia, on the other hand, glycine was capable of generating either analgesia through NMDAR stimulation (obtained with doses <10 nmol) or hyperalgesia through GlyR stimulation and was observed with only the highest dose used. Such apparently different effects obtained by the two endogenous and obligatory coagonists for the NMDAR glycine site raise the need to bear in mind that any possible effect induced by glycine in vivo could represent a cumulative effect resulting from the recruitment of two fast ionotropic receptors, which modulate neural activity in opposing manners (inhibitory or excitatory). It may therefore be

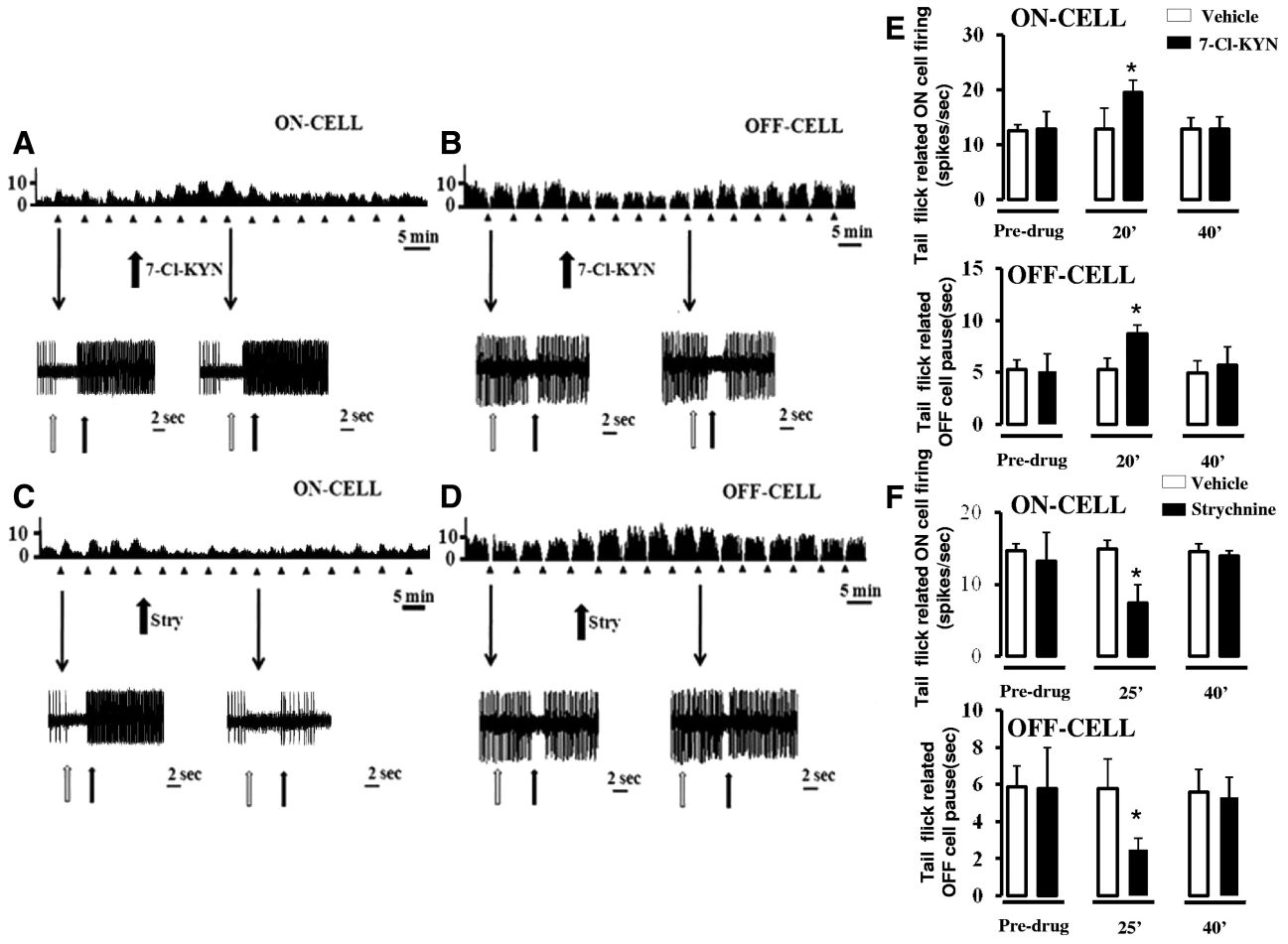

FIG. 7. Examples of ratemeter records that illustrate the effect of intra-PAG microinjections of 7-Cl-KYN (2 nmol) (*A* and *B*) or strychnine (Stry, 2 nmol) (*C* and *D*) on either the ongoing activity or tail-flick–related change in the activity of identified RVM ON cell (*A* and *C*) and ongoing activity or tail-flick–related pause of the identified RVM OFF cell (*B* and *D*). Intra-PAG microinjection of 7-Cl-KYN (2 nmol) increased the ongoing activity and the tail-flick–related frequency of the ON cell (*A*). The same treatment reduced the ongoing activity and increased the tail-flick–related pause of the OFF cell (*B*). Intra-PAG microinjection of strychnine (Stry, 2 nmol) reduced the ongoing activity and the tail-flick–related burst of the ON cell (*C*). The same treatment increased the ongoing activity and decreased the tail-flick–related pause of the OFF cell (*D*). Single oscilloscope traces, indicated by the long black arrows, show the effect of tail-flick stimulation (small gray arrows) and tail-flick reflex (black arrows) on the ON-cell activity or OFF-cell pause before and 20 min after strychnine or 7-Cl-KYN microinjections. Filled triangles indicate tail-flick trials, 1-s bins. Scale bars indicate 5-min or 2-s intervals for ratemeter records or single oscilloscope traces, respectively. *E* and *F*: population data (means ± SE) related to the ON-cell firing (Hz) and OFF-cell pause (s) after microinjection of 7-Cl-KYN (*E*) or strychnine (*F*).

conceivable that, depending on the preferential VL-PAG glycine-recognition site stimulation (i.e., the GlyRs or the NMDARs), the output PAG neurons projecting to the RVM and impinging on the ON and OFF cells may affect the activity of those cells differently with GlyR producing hyperalgesia and NMDAR stimulation producing analgesia. Moreover, depending on the site stimulated, GlyR or glycine site on NMDARs, glycine induced hyperalgesia or analgesia. Importantly, microinjections of 7-Cl-KYN at a higher dose than that used in combination with glycine facilitate nociception and either increase or decrease ON- and OFF-cell activity, respectively. This evidence suggests that the NMDAR glycine site may play a tonic physiological role on pain modulation within the VL-PAG–RVM circuitry. This effect is consistent with our previous studies showing that intra-PAG microinjection of selective NMDA antagonists is capable of enhancing nociceptive behavior in rodents (Palazzo et al. 2001, 2002).

Accordingly, when microinjected into the PAG, glycine or D-serine decreased the ongoing activity of the pronociceptive ON cells and increased the ongoing activity of the antinociceptive OFF cells in the RVM in a way that was prevented by coadministration with 7-Cl-KYN. This effect suggests that stimulation of the NMDAR glycine site modified the activity of PAG output neurons and either decreased or increased the input required to modulate the spontaneous activities of ON and OFF cells. The present finding that 7-Cl-KYN in the VL-PAG blocked glycine- or D-serine–induced antinociception and the related changes in the RVM cell activity suggests that the effects of glycine or D-serine were related to the enhancement of NMDAR-mediated neurotransmission. Since the ON cells may facilitate nociception (Fields et al. 1991), a delayed onset of the ON-cell burst as well as the shortened interval between the onset of the ON-cell burst and tail withdrawal, with either glycine or D-serine, might be expected to be critical events in the occurrence of analgesia (Foo and Mason 2005; Heinricher et al. 1989; Jinks et al. 2004). Indeed, pain transmission and perception are constantly changing: increasing during periods of ON-cell activity and decreasing when OFF cells are active

(Clearly et al. 2008; Mason 2005; Ramirez and Vanegas 1989). Consistent with their effect on ON cells, intra-PAG microinjections of glycine or D-serine delayed the onset of the OFF-cell pause, but shortening its duration and increasing the time between the onset of the pause and tail withdrawal. This is further confirmation of how critical the involvement of both ON and OFF cells is in PAG–RVM-mediated analgesia (Clearly et al. 2008; de Novellis et al. 2005; Heinricher and Tortorici 1994; Maione et al. 2006; McGaraughty and Heinricher 2002).

Significantly, when the highest dose of glycine (20 nmol) was microinjected into the VL-PAG, it increased the ongoing activity of the pronociceptive ON cells and decreased the ongoing activity of the antinociceptive OFF cells in the RVM. This effect appeared with a long latency, making one wonder whether the observed effect was due to the drug diffusion to other nuclei close to the VL-PAG. The anatomical controls performed in the current study revealed that the diffusion radius of 200-nl-volume microinjections was about 0.250–360 mm and VL-PAG has an horizontal axis of 1.36 mm and a vertical one of 1.31 mm in the rat (Paxinos and Watson 1986). Thus in spite of the diffusion the microinjected drug should remain within the VL-PAG. Moreover, when some treatments were intentionally or wrongly (discarded data) performed outside the PAG, they failed to produce any significant change in either behavioral or RVM cell activity. However, it cannot be absolutely excluded that the observed effects could include the involvement and/or the contribution of adjacent areas (i.e., dorsal raphe, dorsolateral PAG).

The effect of the highest dose of glycine was dramatically enhanced by blocking the NMDA glycine site receptor with the lowest dose of 7-Cl-KYN and, accordingly, a hyperalgesic effect was observed in the tail-flick test. This pain facilitatory effect was in turn prevented by strychnine, assuming the presence of functional GlyR in the PAG–RVM circuitries that, similarly to γ-aminobutyric acid type A receptors in the PAG (Moreau and Fields 1986), could be tonically involved in the facilitation of pain transmission. Such evidence would suggest that, depending on the GlyR or NMDA glycine recognition site stimulation, glycine modifies the activity of PAG output neurons affecting the spontaneous activities of ON and OFF cells, thus causing hyperalgesia or analgesia. This possibility seems further strengthened by the fact that, unlike glycine, D-serine, which binds and stimulates the glycine site only on the NMDAR (but not the GlyR) always induced analgesia. Moreover, the different roles played by these amino acids in the PAG derive from the capability of D-serine, compared with glycine, to increase (i.e., 2–3 min postmicroinjection) tail-flick latency more rapidly, although both treatments induced rapid electrophysiological effects. We speculate that these differences could be due to the selective NMDA-related effect of D-serine (i.e., analgesia), compared with glycine, which is in turn also able to modulate its own GlyRs. This makes it possible that the two counteracting effects (i.e., analgesia or hyperalgesia via NMDA or GlyR, respectively) conceal the effect of one another and prevent any change in tail-flick latency, consistent with a delayed inhibition of the pronociceptive ON-cell activity. However, since glycine also induced a 7- to 10-min delayed analgesia, it could be possible that the NMDA-mediated effect may eventually prevail on the GlyR-mediated one.

Consistent with other previous studies (Berrino et al. 1993; Li and Han 2007; Sjöström et al. 2003), these data suggest that the physiological level of glycine in the PAG might be in a critical range, allowing the stimulation of a share of nonsaturated NMDARs in vivo. Indeed, considering that the EC_{50}s of D-serine or glycine for NR1–NR2A (postsynaptic) receptors are known to be higher than those for NR1–NR2B (presynaptic) ones (Kew et al. 1998; Kutsuwada et al. 1992), one could suppose that the postsynaptic (containing NR2A) nonsaturated NMDA subtype receptors would mainly be recruited in vivo by microinjecting glycine or D-serine into the PAG. Moreover, with respect to the GlyRs, we provide in vivo evidence that strychnine induced analgesia per se. However, an unanswered question remains. A recent study has shown that when microinjected into the dorsolateral PAG (D-PAG), glycine was able to induce hyponociception in awake and restrained rats; however, extremely high doses were required and hyperalgesia was not induced (Martins et al. 2008). The differences between the findings of Martins et al. (2008) and those of the current research may be explained by the fact that the two studies were performed in different PAG regions and experimental conditions. Indeed, it should be considered that: *1*) VL-PAG and D-PAG work very differently, as demonstrated either by pharmacological or electrical manipulations, and the same neurotransmitter (i.e., glutamate) can even generate opposite responses (Bandler et al. 1991; Carrive and Bandler 1991); *2*) neural pain circuitry within the VL-PAG is classically modulated by the opioid system, whereas the D-PAG instead seems to be poorly regulated by opioids (Morgan and Liebeskind 1987); *3*) two "pure analgesic regions," both located in the ventral PAG (the dorsal raphe nucleus and the ventrolateral columnar organization of the PAG) have been recognized (Fardin et al. 1984); *4*) we performed the experiments in anesthetized rats by continuous infusion of propofol, which is able to affect both inhibitory and excitatory amino acid neurotransmitter systems (Orser et al. 1994); and *5*) finally, the experiments by Martins et al. (2008) were performed in awake and restrained animals, which implies a stress-induced modification of the nocifensive threshold (i.e., stress-induced analgesia [SIA]). Considering that the D-PAG is critically involved in the SIA genesis it might be possible that such a condition partly contributed to the diminished ability of glycine to induce hyperalgesia.

In conclusion, together with previously reported electrophysiological, pharmacological, and anatomical findings, this study provides evidence indicating that not only the strychnine-insensitive glycine-recognition site on the NMDAR, but also the strychnine-sensitive GlyRs, may have tonic roles in analgesia or hyperalgesia mediated by the VL-PAG. Furthermore, this study highlights the close relationship that exists between the strychnine-sensitive GlyRs and glutamate NMDA-mediated neurotransmissions, adding further complexity to the question of how fast amino acid excitatory and inhibitory neurotransmitters might modulate pain and possibly several other functions within the PAG–RVM pathway.

Since our study and those of others underline the role of glycine and D-serine in the modulation of the descending antinociceptive pathway, new strategies for the development of drugs targeting glycine-recognition site on NMDAR should be aimed at pain control research.

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